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SPINDLY, a tetratricopeptide repeat protein involved in gibberellin signal transduction in *Arabidopsis*

(plant hormones/gibberellin response mutants)

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ABSTRACT **Gibberellins (GAs) are a major class of plant hormones that control many developmental processes, including seed development and germination, flower and fruit development, and flowering time. Genetic studies with *Arabidopsis thaliana* have identified two genes involved in GA perception or signal transduction. A semidominant mutation at the *GIBBERELLIN INSENSITIVE* (*GAI*) locus results in plants resembling GA-deficient mutants but exhibiting reduced sensitivity to GA. Recessive mutations at the *SPINDLY* (*SPY*) locus cause a phenotype that is consistent with constitutive activation of GA signal transduction. Here we show that a strong allele of *spy* is completely epistatic to *gai*, indicating that SPY acts downstream of GAI. We have cloned the *SPY* gene and shown that it encodes a new type of signal transduction protein, which contains a tetratricopeptide repeat region, likely serving as a protein interaction domain, and a novel C-terminal region. Mutations in both domains increase GA signal transduction. The presence of a similar gene in *Caenorhabditis elegans* suggests that SPY represents a class of signal transduction proteins that is present throughout the eukaryotes.**

Gibberellins (GAs) play a role in a number of growth and developmental processes in plants (1, 2). Severe GA-deficient mutants exhibit reduced seed germination, dwarfism of virtually all organs, and aberrant flower, fruit, and seed development. Although the GA biosynthetic pathway has been studied extensively (3), very little is known about GA perception or signal transduction. Genetic analysis has uncovered two classes of mutants that are affected in their response to GA (4). One class consists of dominant or semidominant mutants resembling GA-deficient mutants but exhibiting reduced sensitivity to GA. Mutants of this type have been isolated in maize, wheat, and *Arabidopsis*. The second group, the "slender" mutants, have a recessive overgrowth phenotype that is phenocopied by repeated treatments of wild-type plants with GA and is consistent with a defect causing constitutive GA response. These mutants have been studied in pea, tomato, barley, and *Arabidopsis* (4).

Previously, four mutations at the *SPINDLY* (*SPY*) locus, a "slender" gene in *Arabidopsis*, were isolated from M_2 populations of ethyl methanesulfonate-mutagenized wild-type seeds by selection for germination in the presence of the GA biosynthesis inhibitor, paclobutrazol (5, 6). The phenotypes of *spy* mutants include early flowering, pale green foliage, partial male sterility, and parthenocarpic fruit development. All of these characteristics are observed, but to a lesser extent, in wild-type plants that have been repeatedly sprayed with gibberellic acid (GA_3). In addition, the *spy-1* allele was found to be largely epistatic to the extreme GA-deficient mutant *ga1-2* (5).

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This report describes a new strong allele of *SPY*, *spy-4*, that is tagged by T-DNA insertion. We present the phenotypes of *spy-4* and the *spy-4 ga1-2* and *spy-4 gai* double mutants, and describe the cloning and characterization of the *SPY* gene.

MATERIALS AND METHODS

Genetic Analyses. The *Agrobacterium* seed transformant lines, originally produced by K. Feldmann (7) and distributed though the *Arabidopsis* Biological Resource Center (Columbus, OH), were screened for new *spy* alleles as 49 pools of 100 lines each (stock no. CS3115) as described (5). One new allele, *spy-4*, was recovered and backcrossed to a wassilewskija (WS) plant. Of 107 F_2 seedlings grown on 1× Murashige and Skoog basal salt mixture (MS) plates containing kanamycin (kan) at 50 μg/ml, 80 were kan resistant. This suggested the presence of a single kan locus. Of 43 paclobutrazol-resistant seedlings in the F_2 population, all were kan resistant, but only two-thirds of the nonpaclobutrazol-resistant seedlings were kan resistant. This indicated linkage between kan resistance and paclobutrazol resistance. *spy-4* was backcrossed to WS twice before subsequent phenotypic and genetic analyses. Seeds of the *hy2* alleles *hy2-1* and *ems195* and of *ga1-2* were kindly provided by J. Chory (Salk Institute, La Jolla, CA). *gai* seeds were a gift from M. Koornneef (Agricultural University, Wageningen, The Netherlands).

Gene Cloning. DNA flanking the T-DNA insert was cloned by plasmid rescue (8) and used to probe a genomic library constructed in the binary plant transformation vector pOCA18 (9). Two cosmids, 2118 and 4111, were chosen for further study because the sequences flanking the *spy-4* T-DNA insertion mapped near the center of their inserts. Cosmid 2118 was used to probe a cDNA library constructed in lambda GT22A (J.-S. Hsu and J. S. Gantt, unpublished work). Cosmid 2118 was also transformed into *spy-1* plants as in ref. 10. Cosmid 4111 was used for the restriction fragment length polymorphism (RFLP) mapping shown in Fig. 3.

DNA Sequencing. The 3.5-kb *SPY* cDNA was subcloned from lambda GT22A using polymerase chain reaction (PCR) followed by TA cloning (Invitrogen). Three independent clones were sequenced using the Sequenase Version 2.0 DNA sequencing kit (United States Biochemicals). To sequence the *spy* alleles, reverse transcriptase–PCR was performed on total RNA from the various alleles. PCR products were cloned and sequenced as above. To sequence the genomic DNA, an 8-kb *Xba*I fragment was subcloned from cosmid 2118 into pBluescript KS^+ (Stratagene), and all intron and exon sequences were determined (6479 base pairs). To sequence the intron/

Abbreviations: GA, gibberellin; SPY, SPINDLY; TPR, tetratricopeptide repeat; kan, kanamycin; RFLP, restriction fragment length polymorphism.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U62135).

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exon junctions in the *spy-1* and *spy-2* mutants, genomic DNA was subjected to PCR and the products were sequenced directly.

RESULTS AND DISCUSSION

Isolation of *spy-4*. The *spy-4* mutant was isolated by screening 4900 *Agrobacterium* seed transformant lines that were obtained from the Arabidopsis Biological Resource Center. Seeds were selected for the ability to germinate in the presence of paclobutrazol (5). Pool number CS2635 contained two paclobutrazol-resistant seeds that yielded plants with a spy phenotype. The F_1 seeds produced by crossing *spy-4* and *spy-1* plants germinated in the presence of paclobutrazol and the resulting F_1 plants exhibited a spy phenotype, indicating that *spy-1* and *spy-4* are allelic.

Similar to the previously described spy alleles (5, 6), *spy-4* flowered earlier than wild-type and displayed pale green foliage, partial male sterility, and parthenocarpic fruit development. However, *spy-4* also exhibited obvious partial dominance with respect to flowering time. Under long day conditions, plants heterozygous for *spy-4* flowered earlier than wild-type WS plants but later than homozygous *spy-4* plants (Table 1). Plants heterozygous for *spy-4* did not exhibit partial sterility and seeds heterozygous for *spy-4* failed to germinate in the presence of paclobutrazol, indicating that these traits are fully recessive. The homozygous flowering time phenotype suggests that *spy-4* is the strongest of the five reported *spy* alleles (5, 6).

Double Mutant Analysis. During this study, it was discovered that the previously described *spy-1* line (5) most likely also carries a linked mutation at the *HY2* locus. The evidence for this is as follows: The *HY2* locus maps in the same approximate region as *SPY* (described below). Similar to *spy* mutants, *hy2* mutants are early flowering and have pale green foliage (11). However, *hy2* mutants do not exhibit male sterility and do not germinate in the presence of paclobutrazol (5). Genetic complementation tests indicated that *spy-1* fails to complement *hy2-1*, whereas *spy-4* and *spy-5* fully complement *hy2-1*. Sequencing of the *SPY* cDNA from two *hy2* alleles (*hy2-1* and *ems195*) detected no mutations in the coding region, and RNA blot analysis indicated that there was no change in *SPY* RNA size or abundance in these lines. Finally, the *spy-1* allele exhibits a long hypocotyl phenotype, characteristic of *hy2* mutants, whereas all other spy alleles do not, and *spy-1* has a more severe early flowering phenotype than *spy-2* (5) even though the molecular lesions in these two mutants are very similar (see below). Taken together, these results suggest that the *spy-1* line carries mutations in both the *SPY* and *HY2* genes, and that *SPY* and *HY2* are separate but linked genes affecting partially overlapping aspects of growth and development. Because our original genetic analysis was performed using the *spy-1* line, we sought to confirm these results with the stronger *spy-4* allele.

Double mutants were constructed between *spy-4* and mutants affected in either GA biosynthesis or GA response. Mutations at the *GA1* locus, which encodes the GA biosynthesis enzyme *ent*-kaurene synthetase A (12), block GA biosynthesis early in the synthesis pathway (13, 14). The phenotypes of the strong *ga1-2* mutant include dwarfism, failure to germinate, male sterility, and incomplete petal development. These phenotypes are reversed by applied GAs (15). Fig. 1*A* shows that, as with the weaker *spy-1* allele (5), *spy-4* is largely but not completely epistatic to *ga1-2*. *spy-4 ga1-2* double mutants do not require exogenous GA for seed germination, petal development, and male fertility; however, *spy-4 ga1-2* double mutant plant height is less than that of *spy-4* single mutants. We have also found that, similar to the *spy-1 ga1-2* mutant (5), the *spy-4 ga1-2* double mutant still responds to exogenous GA treatment with an increase in plant height (S.E.J., unpublished observations). These results suggests that *spy-4* plants activate a basal level of GA independent signal transduction, but that they still respond to changes in the levels of active GAs in the plant.

To test the relationship between *spy* mutants and the semidominant GA *insensitive* (*gai*) mutant (16, 17), we constructed the *spy-4 gai* double mutant. Whereas a weak allele, *spy-5*, was only partially epistatic to *gai* (6), *spy-4* is completely epistatic to *gai* (Fig. 1*B*). This unambiguous epistasis allows these two genes to be ordered and suggests that GAI acts upstream of SPY.

In summary, the results from these double mutant analysis together with the spy phenotype suggest that the wild-type SPY product acts as a negative regulator of a portion of the GA signal transduction pathway that is common to all GA responses and that is downstream of both GA biosynthesis and the step affected in the *gai* mutant.

Cloning the *SPY* Gene. The *SPY* gene was cloned by T-DNA tagging. After demonstrating genetic linkage between the kanamycin-resistance locus resident in T-DNA and the *spy-4* phenotype (see *Materials and Methods*), we used plasmid rescue to isolate plant DNA flanking the T-DNA insertion site. Wild-type genomic and cDNA clones spanning this region were then isolated. The 5′-end of one 3.5-kb cDNA was found to be 13 bp downstream of the T-DNA insertion, and this cDNA was presumed to encode the SPY protein. Northern

Table 1. *spy-4* partial dominance

Genotype	Rosette leaf number
Wild-type WS	9.4 ± 0.5
spy-4/+	5.5 ± 0.2
spy-4	2.0 ± 0.0

As an assay for flowering time, the number of rosette leaves produced by the apical meristem before the production of a flowering stem was recorded for plants of the above *spy-4* genotypes. The mean leaf number is shown ± the standard error.

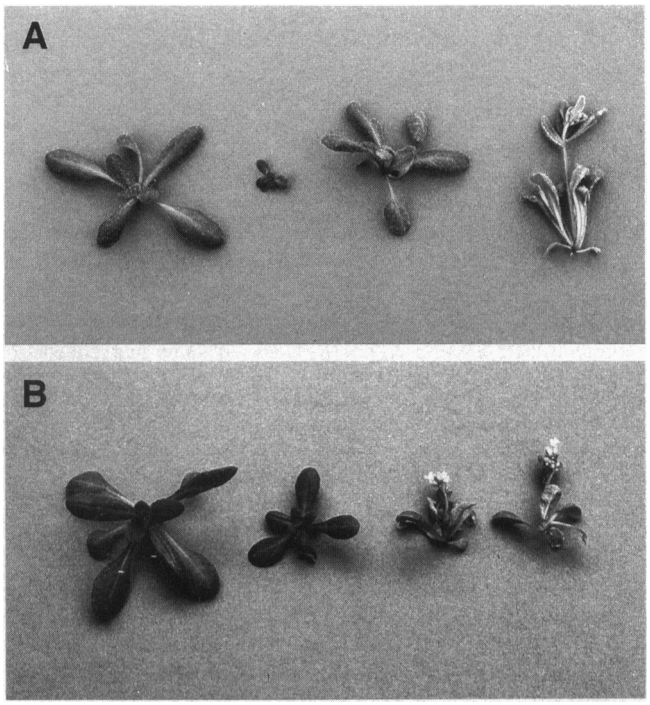

FIG. 1. *spy-4 ga1-2* and *spy-4 gai* double mutants. (*A*) Plants shown from left to right are wild-type L*er*, *ga1-2*, the *spy-4 ga1-2* double mutant, and *spy-4*. Plants were 19 days old. (*B*) Plants shown from left to right are wild-type L*er*, *gai*, the *spy-4 gai* double mutant, and *spy-4*. Plants were 18 days old.

blot analysis indicated that RNA from wild-type and the four EMS alleles contain roughly similar amounts of a 3.5-kb transcript that hybridized to this cDNA, but RNAs hybridizing to this cDNA were not detectable in *spy-4* (data not shown).

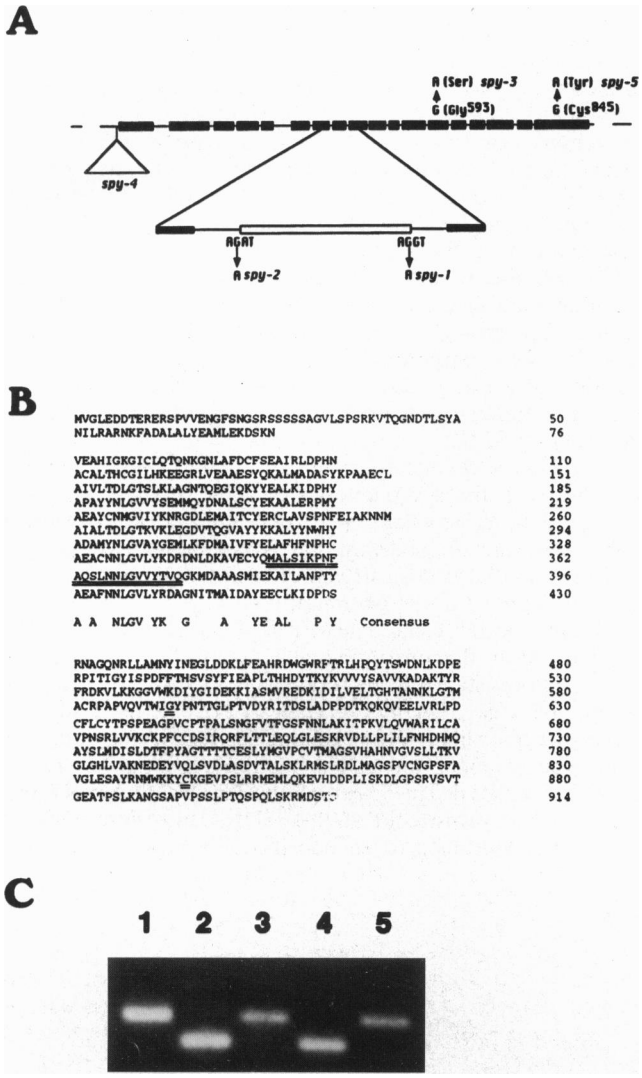

FIG. 2. The *SPY* gene and *spy* mutations. (*A*) Schematic diagram of the *SPY* gene. Broken lines represent the region outside the 3.5-kbp cDNA. Unbroken lines represent introns. Solid bars represent exons. The open bar represents the exon that is skipped in *spy-1* and *spy-2*. Arrows indicate the mutated nucleic acid residues. The triangle represents the T-DNA insertion. (*B*) Deduced amino acid sequence of the SPY protein. The protein is shown in three blocks, the N terminus (residues 1–76), the 10 TPRs (residues 77–430) aligned with each other, and the C terminus (residues 431–914). Below the TPR alignment is a consensus sequence: amino acids are shown if present in at least five of the SPY TPRs. Residues that are double underlined have the following alterations in the mutant alleles: Met^{354} to Gln^{376} are absent in *spy-1* and *spy-2*, Gly593 is converted to Ser in *spy-3*, and Cys^{845} is converted to Tyr in *spy-5*. A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (*C*) RT-PCR products amplified from a region of the *SPY* RNA containing the eighth exon. Lanes: 1, *spy-3*; 2, *spy-1*; 3, *spy-5*; 4, *spy-2*; 5, wild-type. RNA from the wild-type ecotype WS or from the spy mutants was subjected to RT-PCR using the primers 5′-GGCCATAGCTCTGACA and 5′-GCAGCAGAAAGGTTTGCAT. Products were digested with *Hin*dIII, subjected to agarose gel electrophoresis, stained with ethidium bromide, and visualized using UV light. A shorter PCR product was obtained from *spy-1* and *spy-2* RNA than from wild-type RNA. The sizes of the products are consistent with that expected in both the wild-type (416 bp) and the splicing mutants (347 bp).

This correlates with the observation that *spy-4* is the strongest *spy* allele, and suggests that *spy-4* may be an RNA null allele. Comparison between the sequence of the cDNA and the corresponding genomic region indicated that this mRNA is composed of 18 exons that have the potential to encode a 914 amino acid protein (Fig. 2 *A* and *B*). We have confirmed that this mRNA encodes the SPY protein by sequencing *SPY* cDNAs prepared from the *spy-1*, *-2*, *-3*, and *-5* alleles and demonstrating that each contains a mutation. The *spy-3* and *spy-5* mutations cause amino acid substitutions in the C terminus of the protein while cDNAs from both *spy-1* and *spy-2* plants lack the eighth exon (Fig. 2 *A* and *B*). Sequencing of the genomic DNA from these two lines showed that the *spy-1* mutation affects the 5′ exon–intron junction of the eighth exon, whereas *spy-2* affects its 3′ intron–exon junction. Using reverse transcriptase–PCR to amplify the region containing the eighth exon, we have confirmed that this exon is missing in most of the RNAs from *spy-1* and *spy-2* plants (Fig. 2*C*). As predicted, a shorter PCR product was obtained from *spy-1* and *spy-2* RNA than from wild-type RNA. Final confirmation that this gene encodes SPY is provided by the observation that a cosmid clone containing the *SPY* gene (clone 2118) complements the paclobutrazol germination defect of the *spy-1* mutant (see *Materials and Methods*).

Southern blot analysis indicates that *SPY* is a single copy gene (Fig. 3*A*). Using a RFLP present in a *SPY* containing cosmid clone, the *SPY* locus was mapped to the top of chromosome 3 between RFLP markers lAt243 and hsp70-9 (Fig. 3*B*).

Sequence Comparisons. Comparison of the predicted SPY amino acid sequence with protein database sequences indicates that SPY is a tetratricopeptide repeat (TPR) containing protein. The TPR is a 34 amino acid repeated sequence motif found in a number of eukaryotic and prokaryotic proteins (19, 20). Fig. 2*B* shows an alignment of the 10 TPR repeats found in the N terminus of the predicted SPY protein and a con-

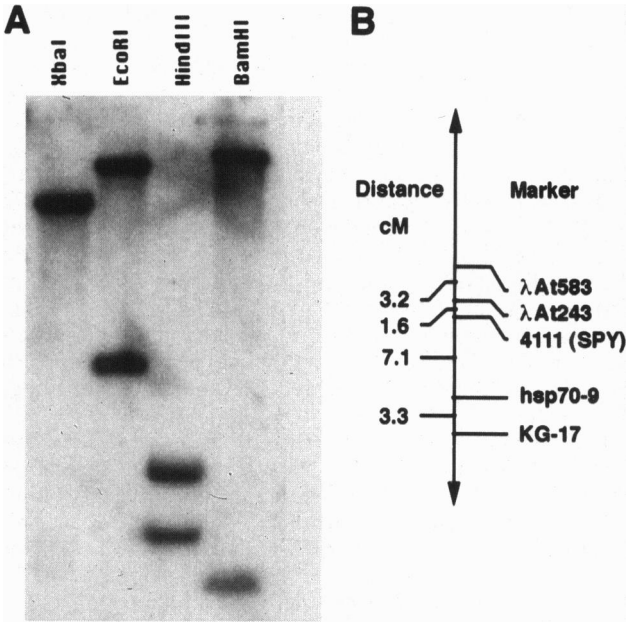

FIG. 3. *SPINDLY* Southern blot analysis and RFLP map position. (*A*) Autoradiogram of a Southern blot containing wild-type Columbia genomic DNA digested with the indicated restriction enzymes and probed with the 3.5-kb SPY cDNA under high stringency conditions. (*B*) The top portion of chromosome 3. A Bgl2 RFLP present in the *SPY* containing cosmid 4111 was mapped on the Landsberg *erecta* X Columbia mapping lines in the laboratory of E. Meyerowitz as in ref. 18. Segregation data from 310 chromosomes were analyzed using MAPMAKER (version 2.0).

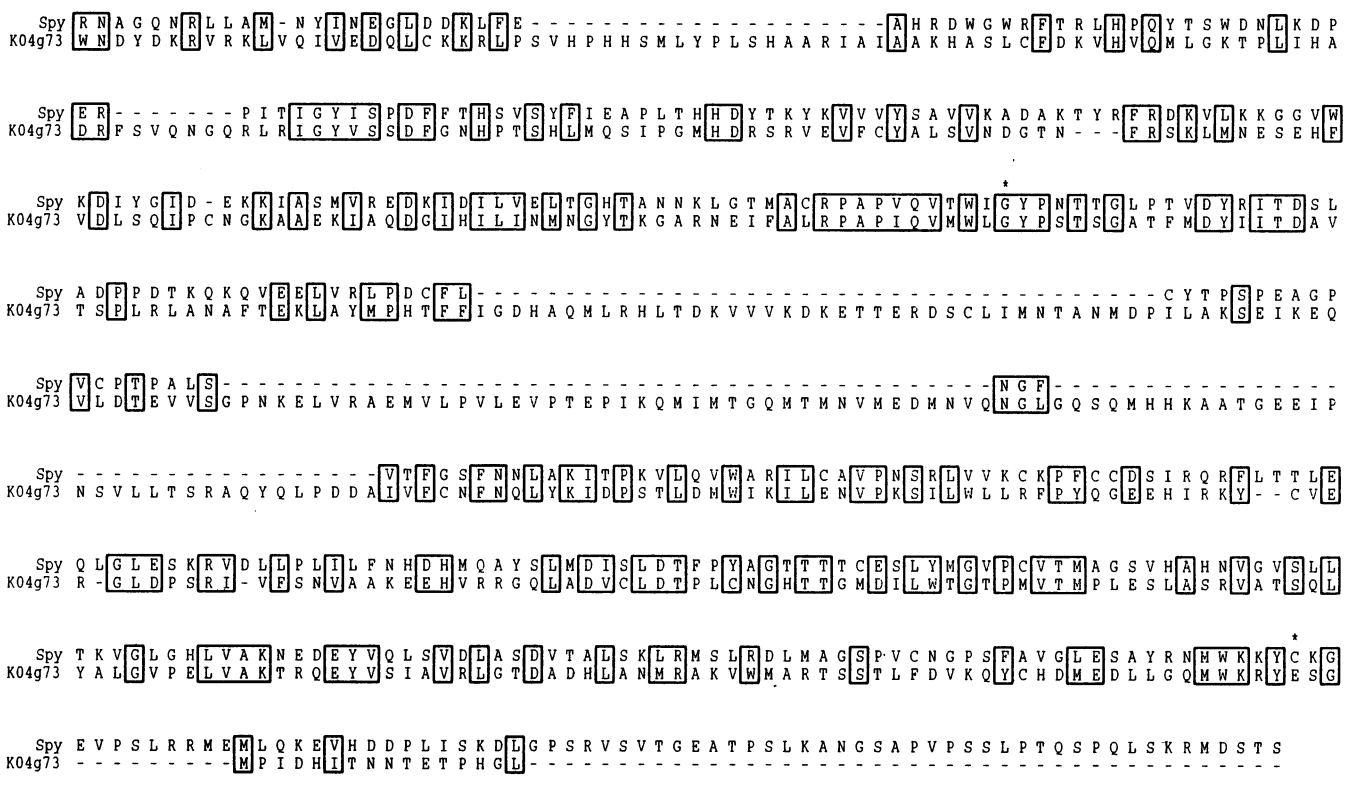

FIG. 4. Alignment of the non-TPR C terminus of SPY (residues 431–914) with that of K04G7.3 (residues 626-1194). Identical or chemically similar amino acids are boxed. Gly^{593} mutated in *spy-3* and Cys^{845} mutated in *spy-5* are indicated with an asterisk. Alignment was performed with the PILEUP and PRETTYPLOT programs (Wisconsin Package, Genetics Computer Group, Madison, WI) using default parameters.

sensus sequence for the 10 repeats. The *spy-1* and *spy-2* mutations that result in skipping of the eighth exon, cause an in-frame deletion of 23 amino acids, which includes the last nine amino acids of the eighth TPR and the first 14 amino acids of the ninth TPR. These TPRs therefore appear to be important for SPY function.

Although *SPY* is only the second TPR gene known in plants (21), it is a member of a growing family of TPR proteins that perform diverse functions. Among the proposed functions for TPR proteins are transcriptional repression, mitochondrial and peroxisomal protein transport, cell cycle regulation, protein kinase inhibition, and heat shock response (19, 20). While there is little in common with the general function of these proteins, they are often found in protein complexes, and it has been proposed that the TPRs form amphipathic alpha-helices that mediate the protein–protein interactions (19, 20, 22–24). TPR proteins are known to interact with other TPR and non-TPR proteins (19). For CYC8, a yeast transcriptional repressor containing 10 TPRs, it was shown that the first three TPRs are necessary and sufficient for direct interaction with another non-TPR protein, TUP1 (23, 24). The CYC8–TUP1 complex is thought to be recruited by specific DNA binding proteins and acts as a transcriptional repressor. Thus, it is likely that SPY acts to suppress GA signal transduction in part by interacting with other proteins through one or more of its TPR domains.

While there are no obvious sequence motifs in the 485 amino acid non-TPR C-terminal region that might indicate a specific biochemical function, the *spy-3* and *spy-5* mutations demonstrate that this domain is also important for normal SPY activity. Comparison of this region with protein database sequences reveals similarity with a predicted protein from *Caenorhabditis elegans*, K04G7.3 (25) (Fig. 4), and less similarity with expressed sequence tags from the blood fluke *Schistosoma mansoni* (GenBank accession no. T14591) and human (GenBank accession no. R76782) (data not shown). The glycine affected in the *spy-3* allele is conserved in K04G7.3, whereas the cysteine affected in *spy-5* is not. Although the function of K04G7.3 is unknown, it also contains an N-terminal TPR region that exhibits the highest level of similarity to the SPY TPR domain. This suggests that SPY and K04G7.3 are members of a new class of regulatory molecules that is likely to be present throughout the eukaryotes. Determining SPY's biochemical function and identifying potentially interacting proteins may aid our understanding of this class of molecules, and in particular, should further our knowledge of the molecular nature of the GA signal transduction pathway.

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